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(54) Title: METHODS OF USING NEUROTROPHIC FACTORS TO ENHANCE NEURONAL GRAFTS

(57) Abstract

The present invention relates to a method of promoting the structural and functional integration of neurons grafted into the central nervous system of a mammal by administering a neurotrophic factor to the central nervous system of the mammal, thereby promoting the structural and functional integration of the grafted neurons. Preferably the neurotrophic factor is administered to the central nervous system during a time period in the ontogenic development of the grafted neurons when the neurons are optimally responsive to the neurotrophic factor. The neurotrophic factor may be selected from the group consisting of NGF, BDNF, NT-3, NT-4/5, and derivatives thereof. Alternatively, the neurotrophic factor may be selected from the group consisting of CNTF, GDNF, and derivatives thereof. At least two neurotrophic factors may also be administered, selected from the group consisting of CNTF, GDNF, NGF, BDNF, NT-3, NT-4/5, and derivatives thereof. Preferably the grafted neurons are dopaminergic neurons and are contained in fetal ventral mesencephalic tissue. Also preferably, the recipient mammal is a human and the graft is made to the striatum of the brain as a treatment for Parkinson's disease.

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METHODS OF USING NEUROTROPHIC FACTORS TO ENHANCE NEURONAL GRAFTS

This application claims priority of United States Application Serial No. 08/427,879 filed April 26, 1995. The present invention relates to methods of using neurotrophic factors to enhance neuronal grafts. More specifically, the present invention relates to a method of promoting the structural and functional integration of neurons grafted into the central nervous system of a mammal by administering a neurotrophic factor to the central nervous system of the mammal. Throughout this application various publications are referenced. The disclosures of these publications in their entireties are hereby incorporated by reference into this application.

BACKGROUND OF THE INVENTION

Neuronal grafting has emerged recently as a potential approach to central nervous system (CNS) therapy. The replacement or addition of cells to the CNS which are able to produce and secrete therapeutically useful substances may offer the advantage of averting repeated drug administration or provide a method of treatment for conditions for which drug therapy is currently nonexistent or suboptimal. Neuronal grafting also offers the potential for replacement of lost neuronal circuits which cannot be accomplished by drug therapy alone. While the concepts and basic procedures of neuronal grafting have long been known, most of the factors that may optimize the survival of grafted cells have only recently been discovered and are still only partially understood. (Bjorklund et al., in Neural Grafting in the Mammalian CNS, p. 709, Elsevier, Amsterdam (1985); Sladek et al., in Neural Transplants: Development and Function, Plenum Press, N.Y. (1984)). Neuronal grafting has reached a level of experimental clinical application in Parkinson's disease.

Parkinson's disease is a disorder characterized by a loss of dopaminergic neurons in the substantia-nigra of the midbrain. The symptoms of the disease include

tremor, rigidity and bradykinesia. It has been suggested that neurodegenerative disorders, such as Parkinson's disease, Alzheimer's disease and amyotrophic lateral sclerosis (ALS), may occur due to the loss or decreased availability of a substance, a neurotrophic factor, specific for a particular population of neurons affected in each disorder. (Phillips, H.S., et al., Neuron 7: 695-702 (1991)).

As used herein, "neurotrophic factor" refers to a substance or combination of substances whose primary function is to increase and/or maintain the survival of a neuronal population, but may also affect the outgrowth of neuron processes and/or the metabolic activity of a neuron. Several neurotrophic factors affecting specific neuronal populations in the central nervous system have been reported. It may be that the loss of such specific neurotrophic factors is responsible for age-related declines in cell survival and/or function. While the cellular source remains unclear, there is evidence to suggest that neurons and glia are both capable of secreting neurotrophic factors. (Lindsay, R.M., et al., TINS 17: 182-190 (1994)).

Parkinson's disease is progressive, but symptoms of this disease can be ameliorated by replacement of dopamine through the administration of pharmacological doses of the precursor for dopamine, L-DOPA. (Marsden, Trends Neurosci. 9:512 (1986); Vinken et al., in Handbook of Clinical Neurology p. 185, Elsevier, Amsterdam (1986)). However, with chronic use of pharmacotherapy, the patient often becomes refractory to the continued effect of L-DOPA. There are many suggested mechanisms for the development of the refractory state, but the simplest is that the patient reaches a threshold of cell loss, wherein the remaining cells cannot synthesize sufficient dopamine from the precursor. In addition, treatment with L-DOPA has no effect on halting the progressive neuronal loss which characterizes the disease.

There is currently no available method for rescuing degenerating dopaminergic neurons in the substantia nigra. In addition, the conditions responsible for the onset of the degeneration of these nerve cells have not been elucidated. Thus,

there is currently no clearly effective cure for Parkinson's disease. However, transplantation of fetal neurons into the central nervous system of neurologically impaired adult animals can ameliorate a wide range of deficits in neural function exhibited by the graft recipients. (Lindvall, O., Transplantation into the human brain: present status and future possibilities. J. Neurol. Neurosurg. Psychiat. Supp.: 39-54 (1989); Lindvall, O. and Bjorklund, A., Acta Neurol. Scand. 126:197-210 (1989); Yurek, D.M. and Sladek, J.R., Jr., Ann. Rev. Neurosci. 13: 415-440 (1990); Freed, W.J., Science 250:1434 (1990); Kordower, J.H., et al., Neural grafting for Parkinson's disease. In: The scientific basis for the treatment of Parkinson's disease, edited by Olanow, C.W. and Lieberman, A.N., New York: Parthenon Publishing, 1992, p. 175-224.) On the basis of such animal studies, engraftment of developing dopaminergic neurons is now being employed as an experimental therapy in the treatment of Parkinson's disease. (Lindvall, O., et al., Science 247: 575-577 (1990); Freed, C.R., et al., Arch. Neurol. 47: 505-512 (1990); Madrazo, I., et al., Arch. Neurol. 47, 1281-1285 (1990); Lindvall, O., et al., Ann. Neurol. 31: 155-165 (1992); Widner, H., et al., N. Eng. J. Med. 327:1556 (1992); Spencer, D.D., et al., N. Eng. J. Med. 327:1541-1548 (1992); Freed, C.R., et al., N. Eng. J. Med. 327: 1549-1555 (1992)).

Several attempts have been made to provide the neurotransmitter dopamine to cells of the diseased basal ganglia of Parkinson's patients by homografting adrenal medullary cells to the brains of affected patients. (Backlund et al., J. Neurosurg. 62:169-173 (1985); Madrazo et al., New Eng. J. Med. 316:831-836 (1987)). The transplantation of other donor cells such as fetal brain cells from the substantia nigra, an area of the brain rich in dopamine-containing cell bodies and also the area of the brain most affected in Parkinson's disease, has been shown to be effective in reversing the behavioral deficits induced by selective dopaminergic neurotoxins. (Bjorklund et al., Ann. N.Y. Acad. Sci. 457: 53-81 (1986); Dunnett et al., Trends Neurosci. 6: 266-270 (1983)).

Although this method of neural replacement shows considerable promise, the extent of functional recovery attained in animal and clinical studies is

incomplete. The partial nature of the recovery has, for the most part, been attributed to the relatively poor rate of survival of the grafted cells and/or their suboptimal synaptic integration with the host brain. (Mahalik, T.J., et al., J. Comp. Neurol. 240: 60-70 (1985); Bolam, J.P., et al., Exp. Brain Res. 68:131-146 (1987); Sladek, J.R., Jr., et al., Brain Res. Bull. 17: 809-818 (1986)). For example, in the context of Parkinson's disease it has been estimated that only 0.5-1% of the total number of transplanted ventral mesencephalic cells both survive and express the desired dopaminergic phenotype. (Bjorklund, A., et al., Acta Physiol. Scand. 522: 9-16 (1983); Brundin, P., et al., Ann. N.Y. Acad. Sci. 495: 473-496 (1987)). In addition, reinnervation of the dopamine-depleted host striatum by the engrafted neurons is limited, and the afferent synaptic integration of grafted dopamine neurons into the host brain is also incomplete. (Freund, T.F., et al., J. Neurosci. 5: 603-616 (1985); Jaeger, C.B., J. Comp. Neurol. 231:121-135 (1985)). Therefore, much of the current research in the field of neural transplantation is focused on developing strategies which improve the survival of engrafted embryonic nerve cells and optimize their appropriate integration into the circuitry of the recipient brain.

It is now well established that trophic factors produced by the developing organism play a critical role in regulating the survival and differentiation of developing neurons. (Snider, W.D. and Johnson, E.M., Ann. Neurol. 26: 489-506 (1989); Whitman, M. and Melton, D.A., Ann. Rev. Cell Biol. 5: 93-117 (1989); Thoenen, H., TINS 14: 165-170 (1991); Lindsay, R.M., et al., TINS 17:182-190 (1994)). Trophic factor expression is dynamic during the course of nervous system development, with specific factors being differentially expressed in various regions of the nervous system, often with distinct temporal periodicities. Consequently, the trophic milieu experienced by neurons which develop in situ will be quite different from that encountered by fetal neurons which are removed from their normal environment and transplanted into a mature or damaged nervous system. Thus, the structural and functional integration of transplanted fetal neurons may be limited, at least in part, by deficiencies in the trophic environment provided by the mature or damaged brain.

Recently, several well characterized trophic factors have been shown to enhance the survival and differentiation of dopamine neurons in tissue culture and/or following transplantation to the anterior chamber of the eye. These trophic factors include fibroblast growth factor, epidermal growth factor, platelet-derived growth factor, transforming growth factor-a, and glial cell derived neurotrophic factor as well as several Nerve Growth Factor (NGF) related neurotrophins. (Casper, D., et al., J. Neurosci. Res. 30: 372-381 (1991); Engele, J., et al., J. Neurosci. Res. 30: 359-371 (1991); Mytilineou, C., et al., Neurosci. Lett. 135: 62-66 (1992); Otto, D. and Unsicker, K., J. Neurosci. 10: 1912-1921 (1990); Pezzoli, G., et al., Movement Disorders 6: 281-287 (1991); Lin, L.H., et al., Science 260: 1130-1132 (1993); Alexi, T. and Hefti, F., Neurosci. 55: 903-918 (1993); Stromberg, I., et al., Exp. Neurol. 124: 401-412 (1993); Giacobini, M.M.J., et al., Dev. Brain Res. 75: 65-73 (1993); Giaeobini, M.M.J., et al., Neurosci. 57: 923-929 (1993)).

Mesencephalic dopamine neurons express both the trkB and trkC neurotrophin receptors and the neurotrophins which act at these receptors, Brain-Derived Neurotrophic Factor (BDNF), Neurotrophin-3 (NT-3) and Neurotrophin-4/5 (NT-4/5) are known to exert trophic effects on fetal dopamine neurons in culture. (Hyman, C., et al., Nature 350: 230-232 (1991); Beck, K.D., et al., Neurosci. 52: 855-866 (1993); Knusel, B., et al., Test 88: 961-965 (1991); Spina, M.B., et al., J. Neurochem. 59: 99-106 (1992); Lindsay, R.M., et al., Exp. Neurol. 124: 103-118 (1993); Hyman, C., et al., J. Neurosci. 14: 335-347 (1994)). Furthermore, BDNF and NT-3 mRNAs are expressed by developing nigral dopamine neurons themselves, with the highest levels of expression being apparent two weeks postnatally. (Friedman, W.J., et al., Eur. J. Neurosci. 3: 688-697 (1991); Gall, C.M., et al., Mol. Cell. Neurosci. 3: 56-63 (1992); Seroogy, K.B. and Gall, C.M., Exp. Neurol. 124: 119-128 (1993)).

BDNF is a member of the neurotrophin family of neuronal survival and differentiation factors which also includes NGF, NT-3 and NT-4/5. BDNF has been shown to enhance the dopamine (DA) uptake of fetal nigral DA neurons in

cell culture (Beck, K.D., et al., Neuroscience 52: 855-866 (1993); Knusel, B., et al., PNAS (USA) 88:961-965 (1991)) and to partially protect DA neurons from the toxic effects of the neurotoxins N-methyl-4-phenylpyridinium ion and 6-hydroxydopamine (6-OHDA) (Hyman, C., et al. Nature 350:230-232 (1991); Spina, M.B. et al., J. Neurochem. 59: 99-106 (1992)). BDNF has also been shown to have a strong supportive effect on the survival of cultured nigral DA neurons. (Beck, K.D., et al., Neuroscience 52: 855-866 (1993); Hyman, C., et al. Nature 350:230-232 (1991); Knusel, B., et al., PNAS (USA) 88:961-965 (1991)). These findings suggested that BDNF might have a survival-promoting effect on grafted DA neurons in vivo.

Thus Sauer, et al. (Brain Research 626: 37-44 (1993)) grafted fetal nigral or basal forebrain tissue to the DA-depleted striatum of unilaterally 6-OHDA-lesioned rats and treated these grafts with either a two-week intraventricular infusion or daily intraparenchymal injection of BDNF, NGF or vehicle. BDNF treatment was shown to enhance the behavioral effect of grafted nigral DA neurons as manifested by a relative reduction in amphetamine-induced turning at two weeks post-grafting. However, this study failed to establish any clear cut difference between treated and control animals in the extent of neurite outgrowth from the grafted DA neurons. Sauer, et al. concluded that although infusion with BDNF produced several behavioral and morphological effects in rats grafted with fetal nigral tissue, it was unable to increase the survival rates of the transplanted dopamine cells.

It is noteworthy that Sauer et al. applied BDNF for only two weeks following transplantation, during a period which was temporally equivalent to the last pre-natal and the first post-natal week of the development of the grafted neurons. In the course of normal development, the dopaminergic fibers within the striatum undergo a dramatic functional and structural alteration during the second through fourth post-natal weeks. (Loizou, L.A., Brain Res. 40, 395-418 (1972); Olson, L., et al., Brain Res. 44: 283-288 (1972); Tennyson, V.M., et al., Brain Res. 46: 251-285 (1972); Coyle, J.T. and Campochiaro, P., J. Neurochem. 27: 673-678 (1976);

Hattori, T. and McGeer, P.L., Exp. Neurol. 38: 70-79 (1973)). Applicants thus decided to determine whether the expression of neurotrophic factors in the neostriatum might be developmentally regulated and further investigated the treatment of grafts of DA neurons with neurotrophic factors in an effort to promote the structural and functional integration of the neurons into the graft recipient.

SUMMARY OF THE INVENTION

The present invention relates to a method of promoting the structural and functional integration of neurons grafted into the central nervous system of a mammal comprising administering a neurotrophic factor to the central nervous system of the mammal, thereby promoting the structural and functional integration of the grafted neurons. Preferably the neurotrophic factor is administered to the central nervous system during a time period in the ontogenic development of the grafted neurons when the neurons are optimally responsive to the neurotrophic factor.

In one embodiment of the invention, the neurotrophic factor is selected from the group consisting of NGF, BDNF, NT-3, NT-4/5, and derivatives thereof. In another embodiment, the neurotrophic factor is selected from the group consisting of CNTF, GDNF, and derivatives thereof. In a further embodiment of the invention, at least two neurotrophic factors are administered and are selected from the group consisting of CNTF, GDNF, NGF, BDNF, NT-3, NT-4/5, and derivatives thereof.

In another embodiment of the invention, the grafted neurons are dopaminergic neurons. The dopaminergic neurons may be derived from a mammal of the same species as the graft recipient or from a mammal of a different species as the graft recipient. In a preferred embodiment, the dopaminergic neurons are contained in fetal ventral mesencephalic tissue. Preferably the recipient mammal is a human and the graft is made to the striatum of the brain as a

treatment for Parkinson's disease.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 - Amphetamine-induced rotational behavior before and after transplant/infusion treatment. Rotational behavior was assessed 3 weeks after the animals received a unilateral 6-OHDA lesion [PREGRAFT] and 3, 4, and 5 weeks after surgical implantation of either transplants/pumps or pumps alone. BDNF [0.75 µg/µl] was delivered at rate of 2.5 µl/hour for 28 days. Bars represent the means for the total number of 360° rotations that occurred during a 90 minute testing period that began immediately after the injection of amphetamine [5.0 mg/kg, i.p.]. Sample size: Transplant+PBS n=15; Transplant+BDNF n=18; (No Transplant)+PBS n=8; and (No Transplant)+BDNF n=8. Error bars, ± standard error of the mean.

* = $p<0.05$ vs. Transplant+BDNF Pregraft mean.

^ = $p<0.05$ vs. Transplant+PBS Pregraft mean.

† = $p<0.05$ vs. Transplant+PBS mean at corresponding time points.

Figure 2 - Photomicrographs of coronal brain sections [35 mm] stained for tyrosine hydroxylase (TH), 5 weeks post-transplantation. BDNF or PBS was infused into the caudate-putamen (CP) for 28 days beginning at the time of transplantation.

(A) Low power photomicrograph of denervated striatum [no transplant] infused with PBS. TH immunoreactivity (ir) is absent.

(B) Low power photomicrograph of transplant (T) infused with PBS. The asterisk indicates the location of the infusion cannula. TH-ir is largely confined to neuronal cell bodies and processes within the transplant, and there is little appreciable re-innervation of the denervated host striatum. (C) Higher power magnification of (B).

(D) Low power photomicrograph of denervated striatum [no transplant] showing that TH-ir is not restored by infusion of BDNF alone.

(E) Low power photomicrograph of a transplant infused with BDNF. Note the extensive distribution of TH-ir within the previously denervated host striatum. (F) Higher power magnification of (E). In grafted animals which had received either PBS (G) or BDNF (H), TH-ir cells and processes are virtually absent from the substantia nigra (SN) and ventral tegmental area (VTA) on the right side, confirming the completeness of the original lesion. The calibration is equivalent to 750 µm for all plates except (C) and (F), where it represents 375 µm. V, lateral ventricle; S, septum.

Figure 3 - Amphetamine induced rotational behavior before and after surgical implantation of transplants/pumps. Amphetamine induced rotational behavior was assessed 3 weeks after 6-OHDA lesion [Pregraft] and 3, 4, 5, 6, 7, 8, 9, and 10 weeks after transplant/pump surgery. BDNF [3.0 µg/µl] was delivered at a rate of 0.5 µl/hour for 28 days. Bars represent the mean of the total number of 360° rotations that occurred during a 90 minute testing period that began immediately after the injection of amphetamine [5.0 mg/kg, i.p.]. Sample size: PBS n=16 and BDNF n=16. BDNF was delivered at a rate of 1.5 mg/hour for 28 days. Error bars, standard error of the mean.

* $p<0.05$ vs. BDNF Pregraft mean.

^ $p<0.05$ vs. PBS Pregraft mean.

† $p<0.05$ vs. PBS mean at corresponding time points.

Figure 4 - Low power photomicrographs of brain sections showing the denvervated/transplanted striatum (right) in animals receiving infusions of PBS (A) or BNDF (B) in EXAMPLE 2. Sections are in the coronal plane and stained for TH, 10 weeks post-transplantation. Note that the striatum on the transplanted side of the BDNF-infused animal shows a normal pattern and near normal density of TH-ir compared to the intact (left) side. In contrast, the TH-ir in the denervated/transplanted striatum of the PBS-infused animal remains sparse. (C) High power photomicrograph of (A) showing transplant and surrounding striatum of PBS-infused animal. TH-ir neurons and neurites are numerous within the transplant (T). Fine as well as coarse TH-ir elements also are apparent

within the host striatum, but are restricted to areas immediately contiguous with the graft.

(D) High power photomicrograph of (B) showing transplant and surrounding striatum in a BDNF-infused animal. In addition to the dense network of TH-ir fibers present within the transplant, TH-ir is distributed widely throughout the host striatum, completely filling the region between the graft and the lateral ventricle (V). As in the intact striatum, fine TH-ir fibers and punctate granules are diffusely distributed throughout the striatal neuropil, while fascicles of white matter remain unstained. Calibration bar equals 1 mm (A,B) or 375 µm (C,D).

Figure 5 - The extent of outgrowth of TH-ir fibers from the grafts was quantified by subjecting stained brain sections to image analysis. The area of striatal neuropil which contained coarse or fine TH-ir processes was measured on the grafted side. Area measurements were averaged for individual animals within each treatment group, and then a treatment average was calculated using the individual animal averages. The top graph shows the average area of the striatum which contained coarse TH-ir fibers and which clearly projected directly from the transplant into the host striatum. The bottom graph shows the average area of the host striatum occupied by fine, diffusely distributed TH-ir fibers and granules. PBS (black bar, n=8) and BDNF (white bar, n=8) treatment groups. Error bars, standard error of the mean. * $p<0.01$ (t-test).

Figure 6 - Northern blot analysis of BDNF mRNA expression in whole adult rat brain (whole brain) and in the rat striatum at various developmental timepoints. Ten micrograms of total RNA per lane were used for each tissue. The BDNF probe identifies two transcripts (1.6 and 4.0 kb).

Figure 7 - Amphetamine induced rotational behavior before and after surgical implantation of transplants/pumps. Amphetamine induced rotational behavior was assessed 3 weeks after 6-OHDA lesion [Pregraft] and 3, 4, 5, 6, 7, 8, 9, and 10 weeks after transplant/pump surgery. Animals were infused with 3.0 µg/ml of BDNF at a rate of 0.5 µl/hour directly into the transplant site for a two week

period that either began immediately after transplant surgery or was delayed for two weeks following surgery. Bars represent the mean of the total number of 360° rotations that occurred during a 90 minute testing period that began immediately after the injection of amphetamine [5.0 mg/kg, i.p.]. Sample size: infusion 3-4 weeks post-transplantation [n=10]; infusion 1-2 weeks post-transplantation [n=9]. Error bars, standard error of the mean.

Figure 8 - Ontogeny of BDNF, dopamine and norepinephrine in rat striatum. Values are means +/- SEM, n= 4-5/group. Adults were 90 days of age. * $p < 0.02$ versus adult for BDNF content.

Figure 9 - Amphetamine induced rotational behavior before and after surgical implantation of transplants/pumps. Bars represent the mean of the total number of 360° rotations that occurred during a 90 minute testing period that began immediately after the injection of amphetamine [5.0 mg/kg, i.p.]. Sample size: infusion 1-2 weeks post-transplantation [n=9]; infusion 3-4 weeks post-transplantation [n=10]; infusion 7-8 weeks post-transplantation [n=9; except where indicated n=4]. Error bars, standard error of the mean.

Figure 10 - Amphetamine-stimulated release of dopamine in denervated/transplanted striatum.

Figure 11 - 6-Hydroxydopamine infusion into transplant site reverses the transplant-mediated correction of amphetamine-induced rotational behavior

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to a method of promoting the structural and functional integration of neurons grafted into the central nervous system of a mammal comprising administering a neurotrophic factor to the central nervous system of the mammal, thereby promoting the structural and functional integration of the grafted neurons. Preferably the neurotrophic factor is

administered to the central nervous system after grafting of the neurons, and during a time period in the ontogenic development of the grafted neurons which corresponds to the time when those neurons, if in their native environment, would normally be exposed to, and would therefore be optimally responsive to, the neurotrophic factor.

In one embodiment of the invention, the neurotrophic factor is selected from the group consisting of NGF, BDNF, NT-3, NT-4/5, and derivatives thereof. In another embodiment, the neurotrophic factor is selected from the group consisting of CNTF, GDNF, and derivatives thereof. In a further embodiment of the invention, at least two neurotrophic factors are administered and are selected from the group consisting of CNTF, GDNF, NGF, BDNF, NT-3, NT-4/5, and derivatives thereof.

In another embodiment of the invention, the grafted neurons are dopaminergic neurons. The dopaminergic neurons may be derived from a mammal of the same species as the graft recipient or from a mammal of a different species as the graft recipient. In a preferred embodiment, the dopaminergic neurons are contained in fetal ventral mesencephalic tissue. Preferably the recipient mammal is a human and the graft is made to the striatum of the brain as a treatment for Parkinson's disease.

Alternatively, dopaminergic neurons could be genetically modified prior to grafting and thus used as carriers for transgenes that endow the grafted cells with properties that may be of therapeutic value. In particular, clonal lines of neural progenitor cells, stem cells or lines derived from neurons or their progenitors may be used. Such cells retain at least some of the features of multipotent neural progenitors after transplantation to the brain and therefore would be suitable for intracerebral grafting and in vivo gene transfer to the CNS. (Martinez-Serrano, A., et al., Use of Immortalized Neural Progenitor Cells For Gene Transfer to the Adult CNS, published in 1994 Neuroscience Short Course - Cell Lines and Transplants, November 13, 1994; Martinez-Serrano, A., et al., Characterization of

Conditionally Immortalized Neural Precursor Cells After Transplantation Into The Adult Rat Striatum, Neuroscience Abstracts No. 205.2 (1994); Renfranz, P.J., et al., Cell 66:713-729 (1991); Onifer, S.M., et al., Transplantation 2: 131-149 (1993); Onifer, S.M., et al., Exp. Neurol. 122 : 130-142 (1993)).

EXAMPLE 1 -

To determine whether BDNF might act to facilitate the innervation of the striatum by developing dopamine neurons, ventral mesencephalic tissue obtained from E14 embryos was transplanted to the striata of adult rats in which the endogenous nigrostriatal dopamine system had been previously ablated on the grafted side by injection of 6-hydroxydopamine (6-OHDA).

6-Hydroxydopamine lesions

Male Sprague-Dawley rats (225-250 gm, Harlan Farms) were given unilateral 6-hydroxydopamine (6-OHDA) lesions of the nigrostriatal pathway; 6-OHDA (Sigma) was dissolved in 0.9% saline (containing 0.2% ascorbic acid) at a concentration of 2.0 µg/µl and stereotactically injected into the nigrostriatal pathway of anesthetized rats at a rate of 1.0 ml/min for 3 min. Each rat received two injections of 6-OHDA: one in the vicinity of the medial forebrain bundle (AP -4.3, ML 1.2, DV -7.5) and the other in the rostral pars compacta of the substantia nigra (AP 4.8, ML 1.5, DV -7.5); all coordinates reported in this study represent millimeter adjustments from bregma (AP, ML) and below the dural surface (DV) with the top of the skull in a flat position.

Ventral Mesencephalic Tissue Grafts

Ventral mesencephalic brain regions were dissected from E13-E15 fetuses obtained from timed pregnant female Sprague-Dawley rats (Harlan Farms) and stored separately in a cold, sterile, calcium and magnesium free buffer (CMF: 0.15 M NaCl, 8.0 mM Na2HPO4, 2.7 mM KCl, 1.5 mM KHPO4, 26.0 mM NaHCO3, 0.1% glucose, 100 mg/ml streptomycin, 2.5 mg/ml fungizone). Recipient animals were anesthetized with sodium pentobarbital and placed in a stereotactic

apparatus. Ventral mesencephalic tissue from a single fetus was aspirated into the blunt end of a 22 gauge spinal needle and stereotactically placed into the denervated striatum of the recipient animal at the following coordinates: AP +0.5, ML +2.5, DV - 5.5.

Intracerebral BDNF Infusions

All working solutions for intracerebral infusions were prepared under sterile conditions. Recombinant metHuBDNF produced by Amgen, Inc. and Regeneron Pharmaceuticals, Inc. was diluted in sterile Dulbecco's phosphate buffered saline (Gibco) to a concentration of 0.75 mg/ml. Either BDNF or sterile PBS alone was loaded into an Alzet 2MLA osmotic minipump (Alzet Corp., flow rate: 2.5 µl/hour for 28 days), a 10 cm piece of PE 60 tubing was attached to the delivery port of the pump, and the pumps were primed in sterile 0.9% saline for several hours prior to implantation. Immediately after transplantation of the ventral mesencephalic tissue, the primed osmotic pump was placed into a subcutaneous pocket on the back. The input port of an osmotic pump connector cannula (Plastic Parts Co.) was attached to the free end of the PE 60 tubing extending from the pump and the cannula lowered stereotactically to a point 0.3 mm dorsal to the transplant site. The cannula was permanently affixed to the skull using dental acrylic and anchor screws that were set into the skull earlier during the surgical procedure. At this concentration and flow rate, BDNF was delivered to the transplant site at a nominal rate of 1.875 µg/hr. Effective delivery of recombinant BDNF from osmotic minipumps has been demonstrated previously. (Altar, C.A., et al., Proc. Natl. Acad. Sci. USA 89: 11347-11351 (1992)).

Beginning at the time of transplantation, BDNF or phosphate buffered saline (PBS) was infused for four weeks into the vicinity of the graft, or into the striata in non-grafted, dopamine depleted control animals. Experimental animals received injections of amphetamine, and asymmetries in locomotor behavior were quantitated as an index of dopamine depletion on the lesioned side relative to the intact side.

Rotational Behavior

In animals with unilateral lesions of the nigrostriatal pathway, asymmetrical locomotor activity, or rotational behavior, is believed to be a result of a hemispheric imbalance in dopamine release. Amphetamine, a potent stimulator of dopamine release, exacerbates the hemispheric imbalance of dopamine release and lesioned animals treated with amphetamine exhibit rotational behavior that is directed away from the intact striatum or, in other words, toward the side of the lesion (ipsilateral rotations), with the number of rotations being directly proportional to the loss of dopamine in the striatum. Treatments which restore dopaminergic activity within the denervated striatum, e.g. neural transplants, attenuate the hemispheric imbalance of dopamine release and consequently attenuate amphetamine-induced rotational behavior. Rotational behavior was monitored using the Videomex V image motion computer system (Columbus Instrument, Inc.). Rats were injected with amphetamine [5.0 mg/kg, i.p.] and placed inside opaque, 16 inch diameter cylindrical chambers with flat bottoms. Chambers were situated directly beneath a video camera and multiple animals were monitored simultaneously. The Videomex V system digitized the image of the animal and calculated the total number of 360° clockwise or counterclockwise rotations that occurred during a 90 minute test period.

Amphetamine-induced rotational behavior was monitored 1 week before transplant surgery [PREGRAFT] and 3, 4, and 5 weeks after surgery. No differences in rotational behavior were exhibited among the treatment groups prior to transplantation. Lesioned animals receiving chronic infusions of PBS or BDNF, but no transplant, showed no significant attenuation in amphetamine-induced rotational behavior at any time during the five week testing period (Figure 1). In contrast, animals receiving transplants plus infusions of PBS exhibited the anticipated progressive reduction in ipsilateral rotations over the course of the experiment. Animals receiving transplants plus infusions of BDNF exhibited a complete attenuation of amphetamine-induced ipsilateral rotational behavior by week 3 and an actual reversal of rotational behavior [net contralateral rotations] in tests conducted 4 and 5 weeks post-transplantation. Though not quantitated, contralateral rotations persisted for

several hours after the end of the testing period in many BDNF treated, grafted animals. This phenomenon was not observed in any grafted animal that received PBS. Contralateral rotations induced by amphetamine continued to increase over time in transplanted animals receiving BDNF, despite the fact that pumps should have exhausted their contents approximately one week prior to the final behavioral test (week 5).

Data Analysis

Rotational scores were subjected to analysis of variance (ANOVA) with repeated measures using the statistical computer program SuperANOVA (Abacus Concepts). The statistical test comprised one between group measure (TREATMENT) with four different levels of treatment: (1) PBS infusions into deneverated striatum, (2) PBS infusions into transplant sites, (3) BDNF infusions into deneverated striatum, or (4) BDNF infusions into transplant sites. The within variable (TIME) comprised 4 repeated measures at the following points: One measure prior to transplant/pump surgery (Pre-graft), and measures made at 3, 4, or 5 weeks after transplant/pump surgery. Statistical analysis of rotational scores revealed a significant TREATMENT effect [$F(3,41)=7.9$, $p<0.001$], a significant effect of TIME [$F(3,123)=6.7$, $p<0.001$], and a significant TREATMENT*TIME interaction [$F(9, 123)=5.5$, $p<0.001$]. The interaction plot is shown in Figure 1 and statistically significant differences between means using a post hoc test for simple main effects are indicated on the figure.

Tyrosine Hydroxylase Immunocytochemistry

At the end of the experiment, animals were anesthetised and intracardially perfused with ice cold saline followed by 4% paraformaldehyde. Brains were removed, post-fixed for 24 hours, and then immersed in 30% sucrose. Free-floating brain sections (35 µm) were immunostained using a monoclonal primary antibody against tyrosine hydroxylase (Chemicon), an avidin-biotin-peroxidase method, (Hsu, S.-M. and Raine, L., J. Histochem. Cytochem. 29:1349-1353 (1981)) and nickel ammonium sulfate intensification of the peroxidase reaction product.

Post-mortem analysis of brain tissue taken from lesioned animals that received infusions of PBS or BDNF alone revealed a near complete absence of tyrosine hydroxylase-immunoreactive (TH-ir) fibers in the striatum on the side of the lesion (Figure 2). Numerous TH-ir perikarya were apparent within the 4 tissue grafts in both PBS and BDNF treated animals. However, a far more extensive and denser reinnervation of the host striatum was apparent in grafted animals that received BDNF compared to those that received PBS. In all treatment groups, negligible TH immunostaining was apparent within the substantia nigra and ventral tegmental area on the side of the right side, confirming that ablation of the endogenous nigrostriatal system was complete.

Thus, infusion of BDNF for four weeks after grafting resulted in a far more extensive and denser reinnervation of the host striatum than was apparent in grafted control animals. Applicants therefore wished to further investigate the ability of BDNF to promote the structural and functional integration of grafted neurons into the central nervous system by monitoring amphetamine-induced rotational behavior for 10 weeks post-transplantation to see if the striatal reinnervation observed in BDNF treated animals might be maintained without further exogenous trophic support.

EXAMPLE 2 -

In the rat, the mature pattern of dopaminergic innervation is maintained throughout adulthood despite the fact that BDNF mRNA is expressed at only very low levels in the striatum at this time. (Maisonpierre, P.C., Neuron 5: 501-509 (1990); Ernfors, P., et al., Neuron 5: 511-526 (1990)). This suggested that, once established, the more complete pattern of striatal reinnervation observed in BDNF treated animals might be maintained without further exogenous trophic support. A second experiment was conducted to test this possibility. As in the first study, PBS or BDNF was infused for 4 weeks beginning at the time of transplantation, but the pumps were then removed from the animals to assure

termination of delivery of BDNF.

Intraparenchymal infusion of PBS or BDNF at the rate of 2.5 µl/hr often produced some degree of cavitation adjacent to the cannula tip. To prevent this, BDNF and PBS were infused as described in the first experiment, except that an osmotic pump with a slower flow rate was used (Alzet 2002 osmotic minipump, 0.5 µl/hour). Brain-derived neurotrophic factor was diluted in sterile PBS to a concentration of 3.0 mg/ml and the BDNF solution or PBS alone was loaded into the pump. At this flow rate and concentration, BDNF was delivered into the transplant site at a rate of 1.50 µg/hr. This dose was selected because it was the smallest amount of BDNF that, when delivered at a flow rate of 0.5 µl/hr, diffused throughout all or most of the striatum as determined by immunostaining of the exogenously delivered protein. Because the model 2002 pump delivers its contents for only 14 days, the first pump was replaced with a fully loaded second pump at the end of the second week in order to acheive continuous delivery for 28 days. This was accomplished by anesthetizing the animal with a halothane-air mixture (1.5% halothane @ 2.0 l/min), making an incision in the midscapular area, cutting the PE 60 tubing, removing the expired pump, and inserting the second fully-loaded pump into the subcutaneous pocket. The PE 60 tubing attached to the delivery port of the second pump was connected to the severed PE 60 tubing attached to the intracerebral cannula using a small piece of 21 gauge stainless steel tubing. Cyanoacrylate adhesive was applied at the tubing junction, and the incision was closed with metal clips. The second pump was removed using this same procedure three weeks later.

Behavioral testing was then continued for an additional 5 weeks (through the 10th post-transplantation week). Figure 3 shows that ipsilateral rotation was reduced in both PBS and BDNF treatment groups by week 3, consistent with the results from the first experiment. Rotational behavior for PBS treated animals then stabilized, and remained at a relatively constant level for the duration of the study. In contrast, BDNF-treated animals exhibited an overcorrection of the initial (ipsiversive) amphetamine-induced rotational asymmetry, as evidenced

by a progressive increase in the rate of contralateral turning during weeks 4, 5, and 6. The rate of contralateral rotation then stabilized, and persisted through week 10, at which time testing was terminated.

Statistical analysis of amphetamine induced rotation in this second experiment comprised one between group measure (TREATMENT) with two different levels of treatment: (1) PBS infusions into transplant sites or (2) BDNF infusions into transplant sites. The within variable (TIME) contained 9 repeated measures: One measure prior to transplant/pump surgery (Pre-graft), and 8 additional measures made 3, 4, 5, 6, 7, 8, 9, or 10 weeks after transplant/pump surgery. Statistical analysis of rotational scores revealed a significant TREATMENT effect [$F(1,30)=10.4$, $p<.01$], a significant effect of TIME [$F(7,210)=26.9$, $p<0.001$], and a significant TREATMENT*TIME interaction [$F(7,210)=5.2$, $p<0.001$]. Interaction plot is shown in Figure 3 and statistically significant differences between means using a post hoc test for simple main effects are indicated on the Figure.

Both BDNF and PBS infused grafts contained large numbers of TH stained neurons (Figure 4). However, fiber outgrowth into the host striatum from BDNF-treated grafts was again far more extensive than that observed in transplants exposed to PBS. The enhanced TH immunostaining in the denervated striatum of grafted animals comprised two morphologically distinct forms: long, coarse processes which often could be traced back directly to the grafts, and fine TH-ir fibers and punctate granules which closely resembled the pattern of TH staining normally seen in the intact striatum.

Quantification of Fiber Outgrowth

Images of brain sections containing TH immunostained transplants were captured via a video frame grabber and stored to computer disk as TIFF files. Image files were subsequently analyzed on a Macintosh IIsi computer using the public domain NIH Image program. Area measurements for coarse TH-ir fibers were compiled from high power images and comprised principally those fibers

which could be traced directly from the graft into the host striatum. Density levels were selected to exclude the following from area calculations: TH-ir cell bodies located on the periphery of the graft, fine TH-ir granules and fibers within the host striatum, and background. Low power (2x) images were used to measure fine TH-ir elements distributed diffusely within the host striatum. For fine TH-ir elements, density levels were selected to exclude the following from the area calculations: TH+ cell bodies, densely stained coarse TH+ fibers, and background. All density measurements were made with the observer blind to the treatment.

Quantitation of fiber outgrowth in immunostained sections showed that BDNF administration increased the area of the host striatum occupied by both coarse and fine TH-ir elements by 3-4 fold relative to PBS treatment (Figure 5). In addition to increasing the overall abundance of TH-ir neurites in the host striatum, BDNF appeared to exert a "tropic" effect on fiber outgrowth from transplants. TH-ir neurites which extended from the grafts into the host brain were preferentially oriented toward the infusion site, and in some cases TH-ir neurons appeared to have migrated from the transplant into contiguous regions of the host striatum which were located nearest the point of infusion.

These results show that upon cessation of exogenous administration of trophic factor to the graft recipient, long term structural and behavioral changes are maintained. This indicates that continued administration of trophic factor to the graft recipient may not be necessary to ensure the structural and functional integration of the neurons grafted into the central nervous system.

Neurochemical Measures of Dopamine Release from Transplants

Dopamine release from transplants was measured by placing a microdialysis probe 1 mm adjacent to the transplant site and collecting dialysate samples under condidons of basal and amphetamine-stimulated release. Basal release of dopamine was found to be the same for both PBS and BDNF treated animals (See Lu et al., 1994, Effect of BDNF on fetal mesencephalic grafts: a microdialysis study,

5th Int. Symp. Neural Transpl. (Abstract)). After the addition of 10 μM amphetamine to the perfusate, we observed a 300% increase in dopamine release relative to basal release in the denervated/transplanted striatum of animals receiving two-week infusions of PBS into the transplant site (Figure 10). In those animals receiving two-week infusions of BDNF into the transplant site, dopamine release after amphetamine treatment was increased 900% relative to basal release (Figure 10). While significant differences in basal dopamine release were not observed, a significant increase in the amount of dopamine release immediately following amphetamine stimulation was observed. These data correlate very well with the observation that BDNF infusions into transplant sites produces an over-correction in amphetamine-induced rotation behavior.

EXAMPLE 3 -

To determine whether the expression of neurotrophic factors in the neostriatum might be developmentally regulated, the levels of BDNF and NT-3 mRNAs in the striata of rats of various ages were determined by Northern blot analysis. Total mRNA was prepared from adult rat brain and from striata dissected from rats of embryonic day 17 (E17), postnatal days 1 (P1), 7 (P7), 14 (P14), and 20 (P20) and adults. Tissue samples were homogenized in 0.3 M LiCl/ 6M urea followed by phenol/chloroform extraction. Ten micrograms of RNA from each sample were electrophoresed on a 1% agarose/formaldehyde gel, followed by capillary transfer to a nylon membrane (MSI). The blots were hybridized overnight at 68°C to either a BDNF probe consisting of a 775 base pair DNA fragment or an NT-3 probe consisting of an 800 base pair DNA fragment labelled with ^{32}P by random oligo priming (Stratagene) as previously described by P. Maisonpierre, et al., Genomic 10: 559 (1991). Ethidium bromide staining of the gel was used to demonstrate equivalent loading of RNA samples.

The results for BDNF, shown in Figure 6, indicate that BDNF mRNA was not detectable before postnatal day 7. However, a dramatic increase in expression was

noted during the second and third postnatal week, corresponding to the period of rapid expansion and maturation of dopaminergic innervation in the rat striatum. These results suggest that the in situ expression of BDNF extends beyond the embryonic stage of development for dopamine neurons and that BDNF is expressed transiently during the development of nigrostriatal dopamine neurons. Similarly, the expression of NT-3 mRNA was observed to exhibit a sharp, transient peak between the first and second postnatal week.

A sensitive and specific enzyme-linked immunoassay (EIA) for BDNF protein was then used to measure the appearance of BDNF in the neostriatum during postnatal ontogeny of the rat and to determine the extent to which the appearance of BDNF coincided with the appearance of dopamine, the principle neurotransmitter released from nigrostriatal neurons.

Striatal tissues were collected at 1, 4, 7, 10, 14, 20, 27, 35, and 45 days after birth from Sprague-Dawley albino rats. The BDNF content of individual striata (n= 4-5 per age group) was determined with a double determinant EIA, in which BDNF extracted from individual striata was captured with a BDNF-specific monoclonal antibody, and a biotinylated, affinity purified rabbit antiserum directed against rhBDNF was used as the reporter antibody (Radke, S., et al., 1996, Brain Res. 709: 122-130). Concentrations of the neurotransmitters dopamine and norepinephrine were measured in aliquots of the striatal homogenate with HPLC (Gamache, P.H., et al., 1993, J. Chromatography 614: 221-220).

The concentration of BDNF protein within striatal tissue was at low levels by one day after birth and increased by about 6-fold to attain peak levels on the 27th day of life (Figure 8; paired t test). These levels on day 27 exceeded those obtained at 45 days of age. Essentially identical patterns were associated with the ontogeny of dopamine in the rat striatum (Figure 8), whereas the neurotransmitter norepinephrine, which is associated with blood vessels in this structure, did not increase in concentration after birth. These data demonstrate the concordant postnatal ontogeny of dopamine nerve terminal ingrowth into the rat striatum,

and the appearance of BDNF, a neurotrophic factor for these same neurons.

EXAMPLE 4 -

It was thought that the failure of the previous study by Sauer, et al., (Brain Research 626: 37-44 (1993)) to establish any clear cut difference between treated and control animals in the extent of neurite outgrowth from the grafted DA neurons was due to BDNF administration to the grafted DA neurons having been stopped prior to the time during which those cells would normally be exposed to endogenous BDNF of striatal origin. To test this hypothesis, applicants decided to investigate the potential effect on functional reinnervation of the striatum caused by delaying the administration of BDNF to grafted DA neurons so that the DA neurons would be exposed to BDNF during a time period in their ontogenic development when the neurons are optimally responsive to the neurotrophic factor.

BDNF was infused continuously into the transplant site at different two-week intervals following transplantation in order to observe whether or not there existed a critical time period when transplanted fetal dopamine neurons might respond more favorably to an exogenous source of BDNF. The age of the fetal tissue (E14) used in these experiments was approximately 1 week prenatal. Therefore, the actual developmental age of transplanted tissue during each infusion period ranged between: (1) -1 to 1 week postnatal for the 0-2 week post-transplantation infusion period, (2) 2-3 weeks postnatal for the 3-4 week post-transplantation infusion period, and (3) 6-7 weeks postnatal for the 7-8 week post-transplantation infusion period.

Ventral mesencephalic tissue obtained from E14 embryos was transplanted to the striata of adult rats in which the endogenous nigrostriatal dopamine system had been previously ablated on the grafted side by injection of 6-hydroxydopamine (6-OHDA) as described above in EXAMPLE 1. Animals were infused with 3.0 µg/ml of BDNF at a rate of 0.5 µl/hour directly into the transplant site for a two week

period that either (1) began immediately after transplant surgery or (2) was delayed for two weeks following surgery. As described above in EXAMPLE 1, the animals received injections of amphetamine, and asymmetries in locomotor behavior were quantitated as an index of dopamine depletion on the lesioned side relative to the intact side. Amphetamine-induced rotational behavior was assessed 3 weeks after 6-OHDA lesion [Pregraft] and 3, 4, 5, 6, 7, 8, 9, and 10 weeks after transplant/pump surgery.

As shown in Figure 7, infusing BDNF directly into the transplant during weeks 3-4 post-transplantation [n=10] had a more profound effect on rotational behavior than infusions during weeks 1-2 post-transplantation [n=9]. The delayed infusion strategy produced a more dramatic and persistent reversal of amphetamine-induced rotational behavior than did the immediate infusion strategy, which produced a slight enhancement of rotational behavior when compared to transplanted animals receiving vehicle infusions.

We then examined and compared the resultant behavioral recovery and morphological development of transplanted dopamine neurons after BDNF was continuously infused into the transplant site during the following two-week intervals: 0-2 weeks post-transplantation, 3-4 weeks post-transplantation, or 7-8 weeks post-transplantation. The first two-week period [0-2 weeks post-transplantation] coincided with the same time period during which Sauer, et al. (Brain Res., 1993, 626: 37-44) intermittently infused BDNF. The second two-week period [3-4 weeks post-transplantation] overlapped with the initial infusion period in which we observed success with stimulating fiber outgrowth from transplants. The last two-week period [7-8 weeks post-transplantation] was a previously untested period that provided us with additional information about the time period in which developing dopamine neurons respond to BDNF treatment.

When BDNF was continuously infused into the transplant site during the first two weeks post-transplantation we observed similar results to those reported by

Sauer et al., that is, rotational behavior was slightly more attenuated in transplanted animals receiving BDNF infusion than in those transplanted animals receiving infusions of PBS, however, morphology of transplants were similar for both groups. At the time BDNF was infused into the transplant the age of the transplanted tissue ranged between -1 to 1 week postnatal. At a similar time period during normal development of the striatum both dopamine content and BDNF protein were at relatively low levels (Figure 8). Delaying the infusion of BDNF into the transplant site until 7-8 weeks post-transplantation did not result in improved transplant function, and some animals even showed an adverse response to these BDNF infusions by subsequently exhibiting increased ipsilateral rotational behavior after administration of amphetamine (Figure 9). On the other hand, when the infusion of BDNF was delayed until 3-4 weeks post-transplantation, we observed an over-correction of amphetamine-induced rotational and enhanced fiber outgrowth (Figure 9). Age of transplanted tissue ranged between 2-3 weeks postnatal during the infusion period. This is the same time period when both dopamine and BDNF show a tremendous surge in activity during the course of normal striatal development (Figure 8). These results further suggest that transplanted immature dopamine neurons are more responsive to the neurotrophic action of BDNF during a critical period of neuronal development which ordinarily occurs postnatally.

These data support the view that there is a critical period of neuronal development that occurs after the embryonic stage of development during which transplanted immature dopamine neurons are more responsive to the neurotrophic action of BDNF. Based on these results, neurotrophic factors administered to the central nervous system to promote the structural and functional integration of grafted neurons should be administered during a time period in the ontogenic development of the grafted neurons when the neurons are optimally responsive to the neurotrophic factor. Knowing that this critical period of time exists for promoting the structural and functional integration of grafted neurons, one of skill in the art can readily determine the optimally responsive period and administer the neurotrophic factor accordingly.

EXAMPLE 5 -

Brain-derived neurotrophic factor is known to exert neurotrophic effects upon not only dopaminergic neurons, but also on GABAergic neurons (Hyman, et al., 1994, J. Neurosci. 14: 335-347) and possibly serotonergic neurons. Moreover, BDNF is known to modulate neuropeptide in both the intact and denervated striatum (Sauer et al., 1994, NeuroReport 5: 609-612). Therefore, it is conceivable that the effects of BDNF on behavior, and more specifically rotational behavior, may also reflect changes in non-dopaminergic systems. To test this hypothesis we duplicated our initial finding that BDNF+Transplants produce an over-correction of amphetamine-induced rotational behavior in rats and allowed the transplants to develop and integrate with the host striatum for a period of 7 weeks post-transplantation. After the seventh week, 25 µg of 6-OHDA dissolved in 3.0 µl of 0.2% ascorbic acid was infused into the transplant site as a means to remove the dopaminergic component of the transplant. This treatment essentially reverses the transplant-mediated correction of amphetamine-induced rotational behavior and we observe amphetamine-induced rotational scores that are similar to scores obtained prior to transplantation (Figure 11). This would suggest that, at least for amphetamine-induced rotational behavior, the over-correction of ampehtamine-induced rotational behavior observed in BDNF/Transplant animals is primarily related to dopaminergic components of the transplant. Furthermore, our initial studies demonstrated that infusions of BDNF in the denervated striatum alone do not affected amphetamine-induced rotational behavior. Therefore, the overcorrection of amphetamine-induced rotational behavior does not appear to be related to the effect of BDNF on the denervated host striatum alone, occurs only in transplanted animals receiving infusions of BDNF into the transplant site, and is reversed when dopaminergic components of the transplant are eliminated by an infusion of a dopamine neurotoxin into the transplant site. The results of these studies infer that there is a strong dopaminergic component involved in the mediation of the rotational

response to amphetamine.

The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and accompanying figures. Such modifications are intended to fall within the scope of the appended claims.

What is claimed is:

1. Use of a neurotrophic factor in the manufacture of a medicament for use in a method of promoting the structural and functional integration of neurons grafted into the central nervous system of a mammal, wherein the medicament is administered to the central nervous system of the mammal.

2. Use according to claim 1 wherein the method comprises administering the neurotrophic factor to the central nervous system during a time period in the ontogenic development of the grafted neurons when the neurons are optimally responsive to the neurotrophic factor.

3. Use according to claim 1 or 2 wherein the neurotrophic factor is selected from NGF, BDNF, NT-3, NT-4/5, CNTF, GDNF; and derivatives thereof.

4. Use according to any one of the preceding claims wherein the grafted neurons are dopaminergic neurons.

5. Use according to any one of the preceding claims wherein the dopaminergic neurons are derived from a mammal of the same species as the graft recipient.

6. Use according to any one of claims 1 to 4 wherein the dopaminergic neurons are derived from a mammal of a different species than the graft recipient.

7. Use according to any one of the preceding claims wherein the dopaminergic neurons are contained in fetal ventral mesencephalic tissue.

8. Use according to claim 7 wherein the fetal ventral mesencephalic tissue is taken from a mammal of the same species as the graft recipient.

9. Use according to claim 7 wherein the fetal ventral mesencephalic tissue is taken from a mammal of a different species than the graft recipient.

10. Use according to any one of the preceding claims wherein the graft recipient mammal is a human.

11. Use according to any one of the preceding claims wherein the method comprises making a graft to the striatum as a treatment for Parkinson's disease.

12. Use according to any one of the preceding claims wherein the neurotrophic factor is BDNF.

13. Use of a first neurotrophic factor according to any one of the preceding claims, in combination with at least one other neurotrophic factor selected from CNTF, GDNF, NGF, BDNF, NT-3, NT4/5, and derivatives thereof.

14. A method of promoting the structural and functional integration of neurons grafted into the central nervous system of a mammal comprising administering a neurotrophic factor to the central nervous system of the mammal, thereby promoting the structural and functional integration of the grafted neurons.

15. The method of claim 14, wherein the neurotrophic factor is administered to the central nervous system during a time period in the ontogenic development of the grafted neurons when the neurons are optimally responsive to the neurotrophic factor.

16. The method of claim 14 or 15 wherein the neurotrophic factor is selected from the group consisting of NGF, BDNF, NT-3, NT-4/5, CNTF, GDNF; and derivatives thereof.

17. The method of any one of claims 14 to 16 wherein the grafted neurons are

dopaminergic neurons.

18. The method of any one of claims 14 to 17 wherein the dopaminergic neurons are derived from a mammal of the same species as the graft recipient.

19. The method of any one of claims 14 to 18 wherein the dopaminergic neurons are derived from a mammal of a different species than the graft recipient.

20. The method of any one of claims 14 to 19 wherein the dopaminergic neurons are contained in fetal ventral mesencephalic tissue.

21. The method of claim 20 wherein the fetal ventral mesencephalic tissue is taken from a mammal of the same species as the graft recipient.

22. The method of claim 20 wherein the fetal ventral mesencephalic tissue is taken from a mammal of a different species than the graft recipient.

23. The method of any one of claims 14 to 22, wherein the graft recipient mammal is a human.

24. The method of any one of claims 14 to 23 in which the graft is made to the striatum as a treatment for Parkinson's disease.

25. The method of any one of claims 14 to 24 wherein at least one other neurotrophic factor selected from CNTF, GDNF, NGF, BDNF, NT-3, NT-4/5; and derivatives thereof is administered.

26. The method of any one of claims 14 to 25 wherein the neurotrophic factor is BDNF.

27. Use according to claim 1 substantially as hereinbefore described, with

reference to any one of the Examples.

28. The method of claim 14 substantially as hereinbefore described, with reference to any one of the Examples.

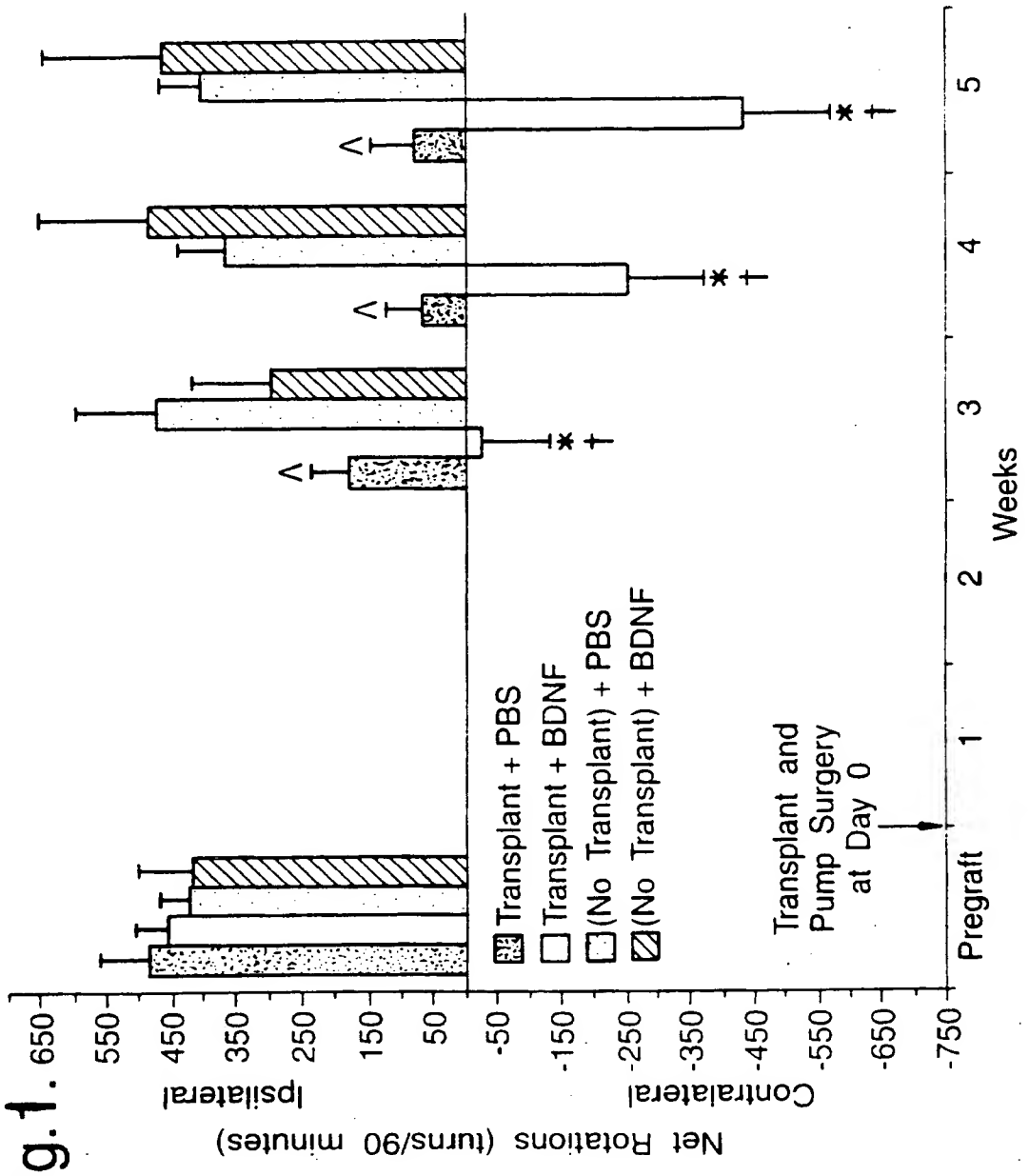

Net Rotations (turns/90 minutes)
Ipsilateral
Contralateral
650
550
450
350
250
150
50
-50
-150
-250
-350
-450
-550
-650
-750
Transplant + PBS
Transplant + BDNF
(No Transplant) + PBS
(No Transplant) + BDNF
Transplant and
Pump Surgery
at Day 0
Pregraft
1
2
3
4
5
Weeks

Fig. 1.

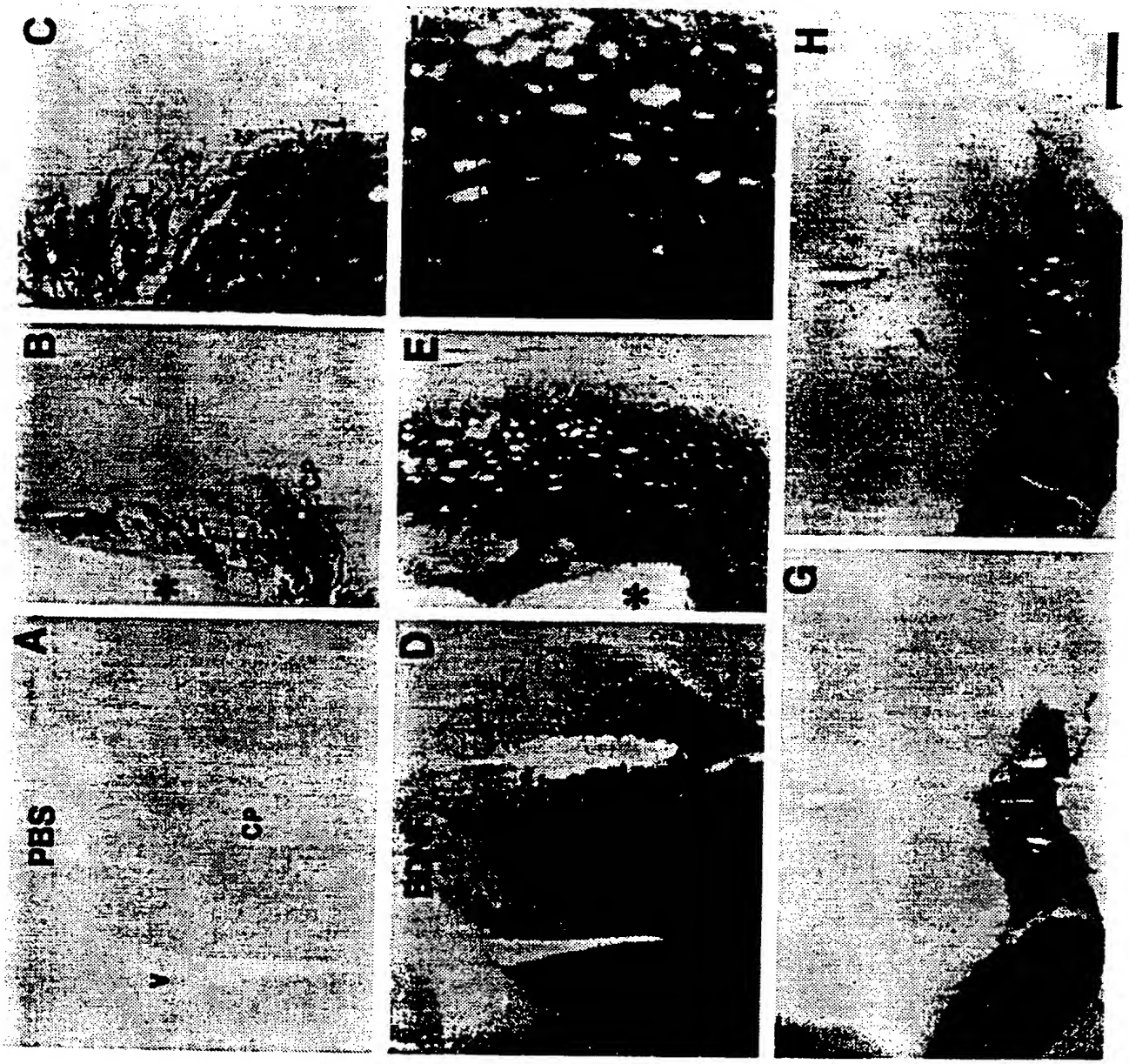


Fig.2.

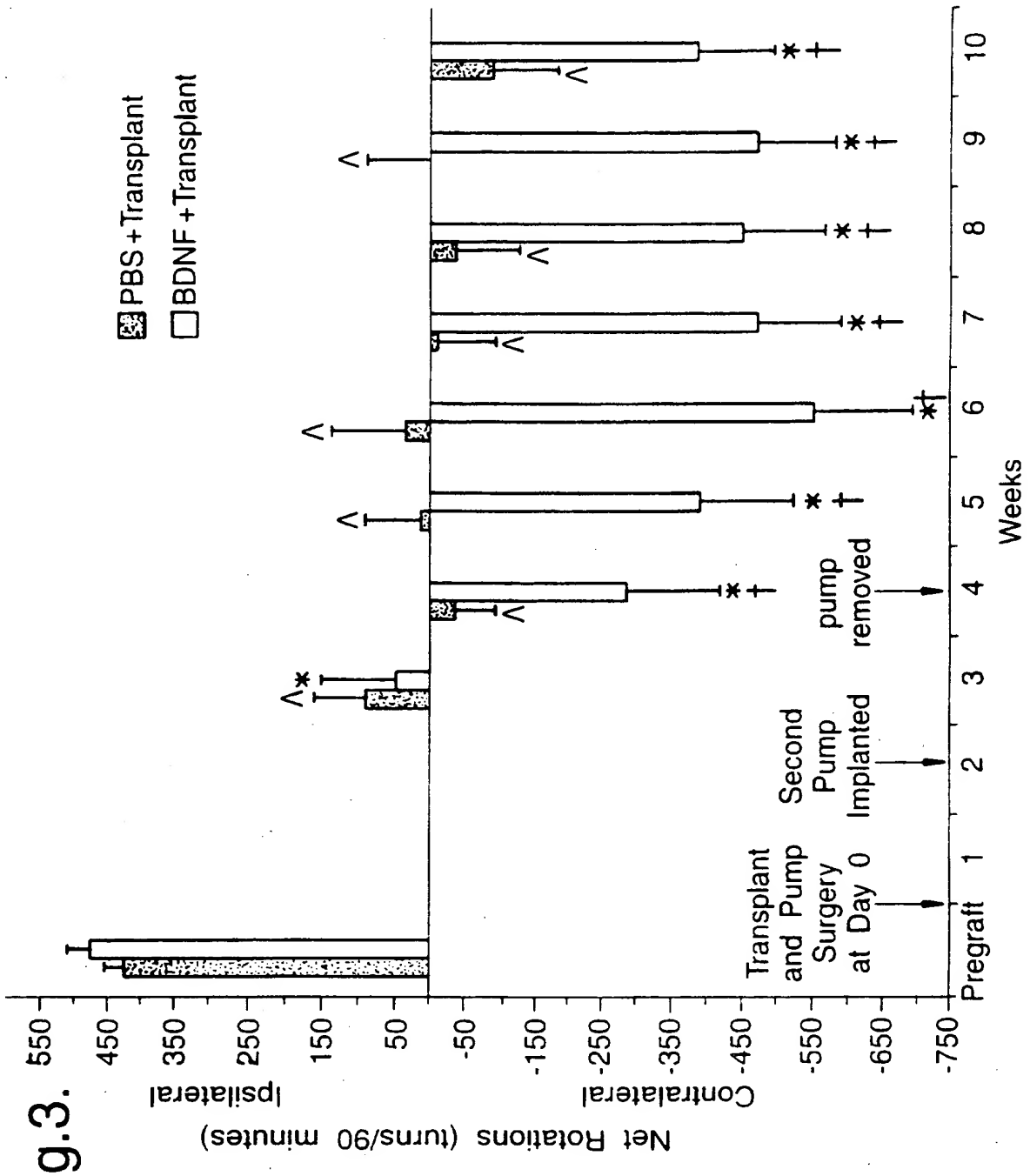

PBS + Transplant
BDNF + Transplant
Net Rotations (turns/90 minutes)
Ipsilateral
Contralateral
550
450
350
250
150
50
-50
-150
-250
-350
-450
-550
-650
-750
Pregraft
1
2
3
4
5
6
7
8
9
10
Weeks
Transplant and Pump Surgery at Day 0
Second Pump Implanted
pump removed

Fig.3.

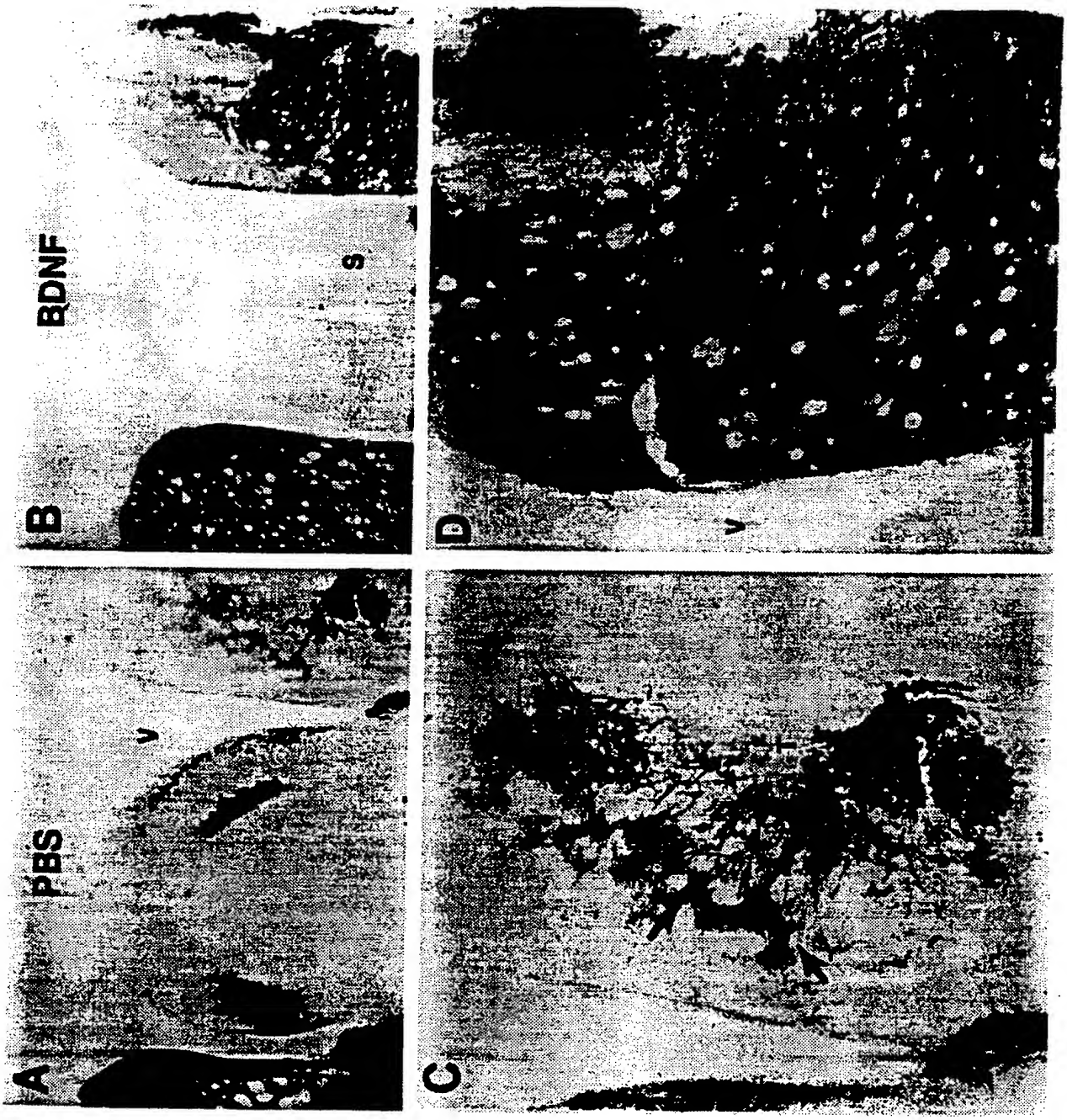


Fig.4.

Fig.5.

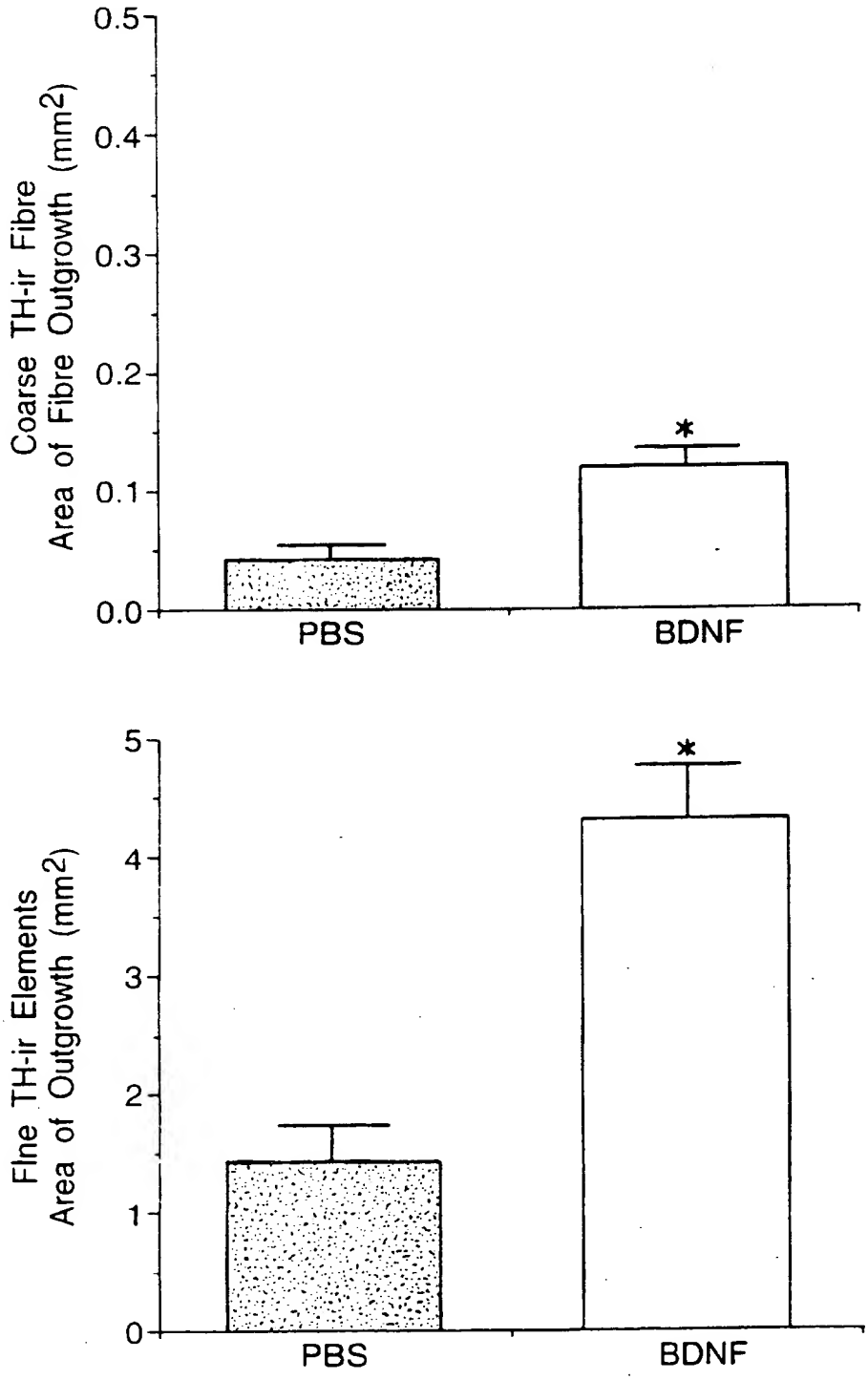

Coarse TH-ir Fibre
Area of Fibre Outgrowth (mm2)
0.5
0.4
0.3
0.2
0.1
0.0
*
PBS
BDNF
Fine TH-ir Elements
Area of Outgrowth (mm2)
5
4
3
2
1
0
*
PBS
BDNF

Fig.6.

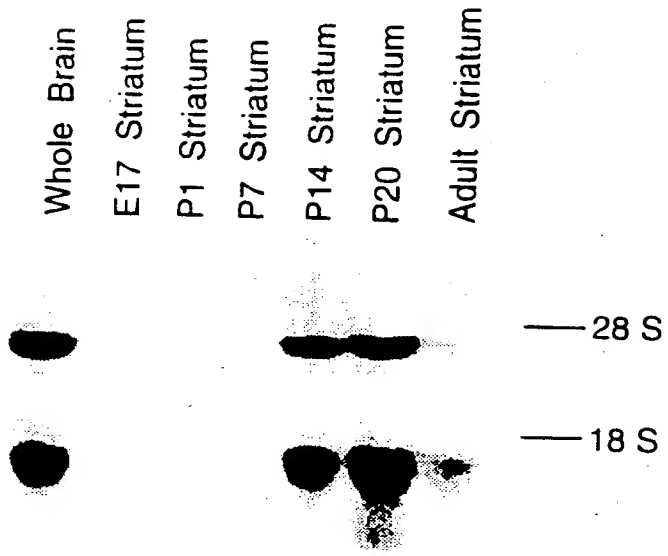

Whole Brain
E17 Striatum
P1 Striatum
P7 Striatum
P14 Striatum
P20 Striatum
Adult Striatum
28 S
18 S

Fig.7.

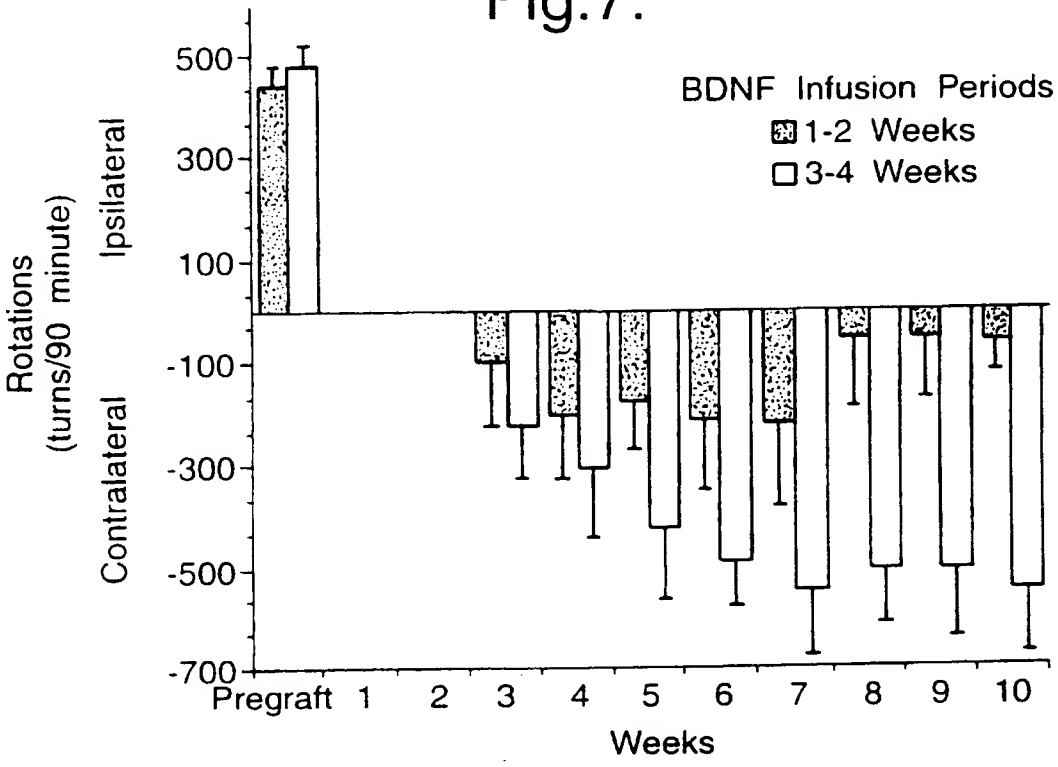

BDNF Infusion Periods
1-2 Weeks
3-4 Weeks
Rotations
(turns/90 minute)
Ipsilateral
Contralateral
500
300
100
-100
-300
-500
-700
Pregraft
1
2
3
4
5
6
7
8
9
10
Weeks

Fig.10.

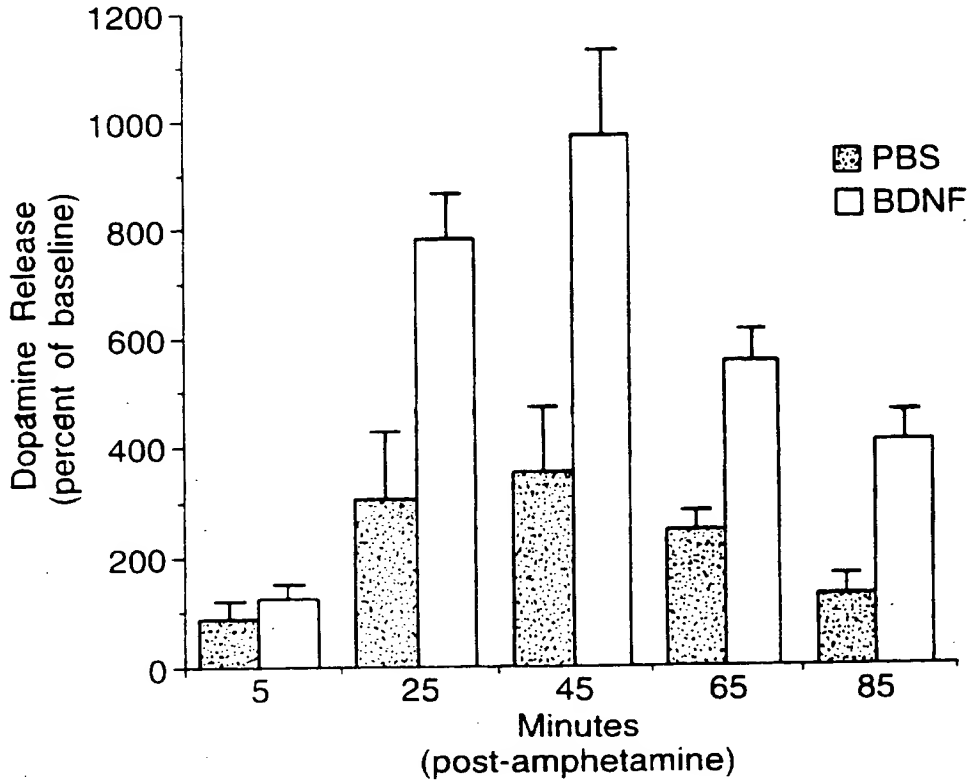
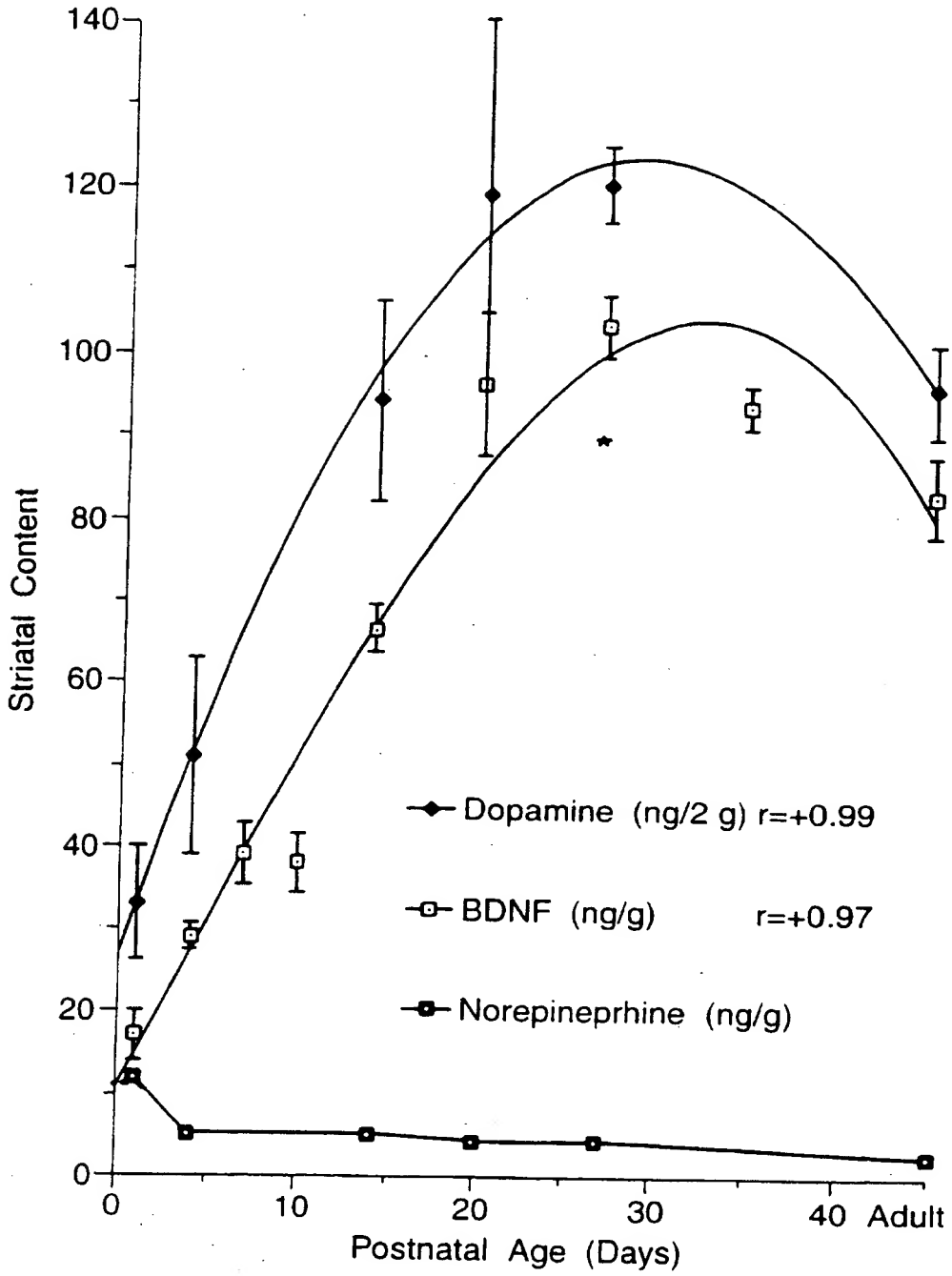

PBS
BDNF
Dopamine Release
(percent of baseline)
1200
1000
800
600
400
200
0
5
25
45
65
85
Minutes
(post-amphetamine)

Fig.8.


140
120
100
80
60
40
20
0
Striatal Content
0
10
20
30
40 Adult
Postnatal Age (Days)
Dopamine (ng/2 g) r=+0.99
BDNF (ng/g) r=+0.97
Norepineprhine (ng/g)

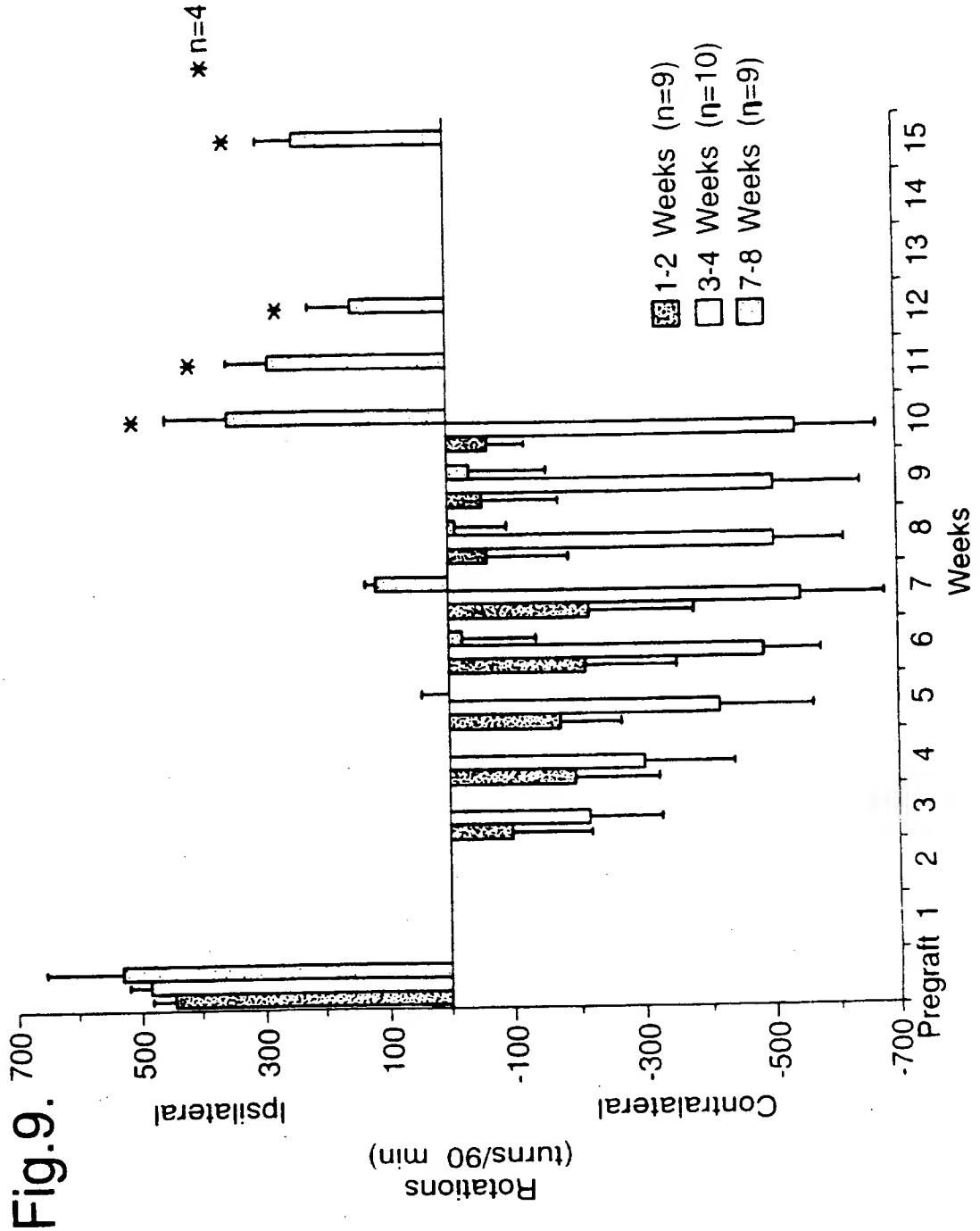

*n=4
1-2 Weeks (n=9)
3-4 Weeks (n=10)
7-8 Weeks (n=9)
Weeks
Pregraft 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
700 500 300 100 -100 -300 -500 -700
Ipsilateral
Contralateral
Rotations (turns/90 min)

Fig.9.

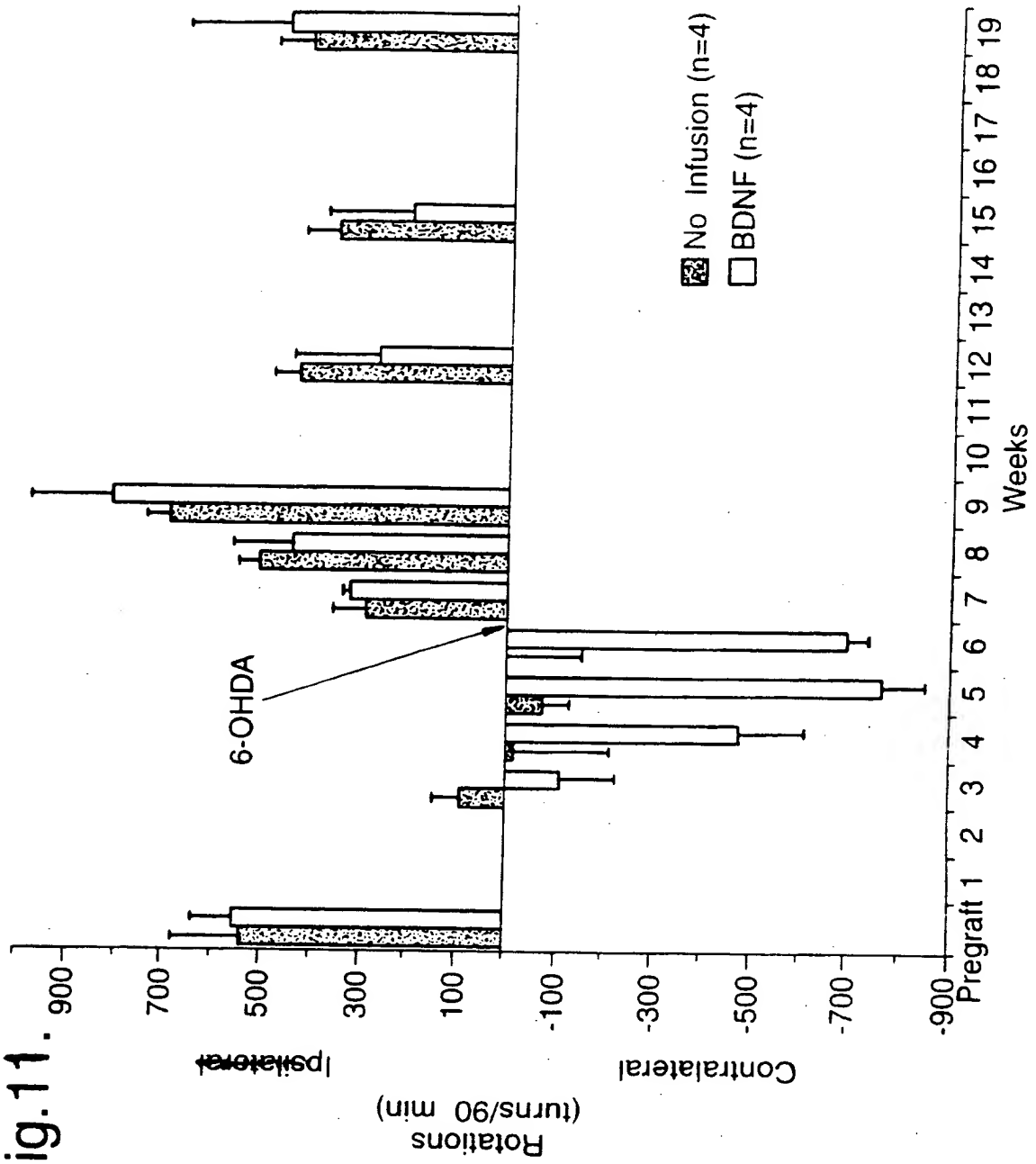

Rotations
(turns/90 min)
Ipsilateral
Contralateral
900
700
500
300
100
-100
-300
-500
-700
-900
Pregraft 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
Weeks
No Infusion (n=4)
BDNF (n=4)
6-OHDA

Fig.11.

INTERNATIONAL SEARCH REPORT

Int onal Application No
PCT/US 96/05814

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K38/18

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,94 03199 (REGENERON PHARMACEUTICALS, INC.) 17 February 1994 see the whole document ---	1-28
X	KEYSTONE SYMPOSIUM ON THE MOLECULAR AND CELLULAR BASIS OF HUMAN NEURODEGENERATIVE DISEASE, BRECKENRIDGE, COLORADO, USA, APRIL 3-9, 1995. JOURNAL OF CELLULAR BIOCHEMISTRY SUPPLEMENT 0 (21B). 1995. 109, XP002010193 KANG U J ET AL: "Brain - derived neurotrophic factor (BDNF)-secreting grafts protect dopaminergic neurons in vivo from partial 6-hydroxydopamine (6-OHDA) lesions." see the whole document --- -/--	1-28

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

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Date of the actual completion of the international search	Date of mailing of the international search report
6 August 1996	09.08.96

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Moreau, J

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CURRENT OPINION IN NEUROBIOLOGY 4 (5). 1994. 752-757, XP000578042 LINDVALL O ET AL: "Clinical application of cell transplantation and neurotrophic factors in CNS disorders." see the whole document ---	1-28
A	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 89, no. 23, 1 December 1992, WASHINGTON US, pages 11347-11351, XP002010194 ALTAR C.A. ET AL.: "Brain-derived neurotrophic factor augments rotational behavior and nigroastriatal dopamine turnover in vivo" cited in the application see the whole document ---	1-28
P,X	DATABASE BIOSIS BIOSCIENCES INFORMATION SERVICE, PHILADELPHIA, PA, US YUREK D M ET AL: "Optimal effects of exogenous BDNF on grafts of fetal dopamine neurons coincides with the ontogenic period when dopamine content and BDNF expression increase within the striatum." XP002010195 see abstract & 25TH ANNUAL MEETING OF THE SOCIETY FOR NEUROSCIENCE, SAN DIEGO, CALIFORNIA, USA, NOVEMBER 11-16, 1995. SOCIETY FOR NEUROSCIENCE ABSTRACTS 21 (1-3). 1995. 1562, ---	1-28
P,X	DATABASE BIOSIS BIOSCIENCES INFORMATION SERVICE, PHILADELPHIA, PA, US WIEGAND S J ET AL: "Effects of BDNF infusion on locomotor behavior and dopamine neurite outgrowth from fetal mesencephalic transplants." XP002010196 see abstract & 25TH ANNUAL MEETING OF THE SOCIETY FOR NEUROSCIENCE, SAN DIEGO, CALIFORNIA, USA, NOVEMBER 11-16, 1995. SOCIETY FOR NEUROSCIENCE ABSTRACTS 21 (1-3). 1995. 1562, ---	1-28
1	-/--	

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	EXP. NEUROL. (1996), VOLUME DATE 1996, 137(1), 105-18, 1996, XP000578016 YUREK, DAVID M. ET AL: "BDNF enhances the functional reinnervation of the striatum by grafted fetal dopamine neurons" see the whole document ---	1-28
P,X	J. NEUROSCI. (1995), 15(12), 7810-20, 1995, XP000578099 LEVIVIER, MARC ET AL: "Intrastriatal implantation of fibroblasts genetically engineered to produce brain - derived neurotrophic factor prevents degeneration of dopaminergic neurons in a rat model of Parkinson 's disease" see the whole document -----	1-28

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INTERNATIONAL SEARCH REPORT

[redacted]onal application No.
PCT/US96/05814

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claims 14-26 and 28 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 96/05814

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9403199	17-02-94	AU-B- 4995193	03-03-94
		ZA-A- 9305648	29-08-94